Babesia microti CYSTEINE PROTEASE-1 AS A TARGET FOR VACCINE DEVELOPMENT

A Thesis

by

ALLISON MELISSA JAMES

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2005

Major Subject: Veterinary Parasitology

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Approved by:

Chair of Committee,	Patricia Holman
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ABSTRACT

Babesia microti Cysteine Protease-1 as a Target for Vaccine Development.

(August 2005)

Allison Melissa James, B.S., University of California at Irvine

Chair of Advisory Committee: Dr. Patricia Holman

Babesia species have a worldwide distribution, affecting a wide range of mammalian hosts. The major route of transmission is inoculation by an infected *Ixodid* tick. *Babesia* species of major economic concern are those that cause bovine and equine babesiosis. Historically, bovine *Babesia* species, *Babesia bovis* and *Babesia bigemina* caused significant economic losses in the United States in the 1860's, as thousands of cattle died. Also, outbreaks of equine babesiosis, caused by *Babesia equi* or *Babesia caballi*, have occurred in the United States resulting in the death of some horses and millions of dollars in losses. A constant risk of reinfection with bovine and equine *Babesia* species exists, as stray and smuggled animals from Mexico, where bovine babesiosis is endemic, may carry infected ticks as they cross the border, and, thousands of horses from *B. equi*- and *B. caballi*-endemic regions are imported through Florida every year.

Vaccines have been developed for a number of *Babesia* species, none of which result in sterile immunity. The live attenuated vaccine is the most commonly used vaccine against *Babesia* species. However, the basis for the vaccine is to maintain a carrier state

in order to prevent disease. Other vaccine designs have been developed to invoke protection without a carrier state but have been unsuccessful.

It has been shown that the cysteine protease is important in the life cycle of a number of parasitic organisms, making it a good target for vaccine development. The vaccine design for this study incorporated the cysteine protease of *Babesia microti*. *Babesia microti* naturally infects *Peromyscus leucopus* (white-footed mouse) and is the major cause of human babesiosis in the United States. Using *B. microti* in the vaccine design allowed for the use of a mouse model to determine whether the cysteine protease of other economically important *Babesia* species may make a good vaccine target. The vaccine design incorporated a prime-boost strategy, priming with DNA encoding the cysteine protease and boosting two times with either DNA encoding the cysteine protease or cysteine protease peptide, followed by parasite challenge. Analysis of daily percent parasitemias, packed cell volume, and seroconversion of all groups revealed that a protective immune response against *B. microti* was not elicited by this vaccine strategy.

DEDICATION

I dedicate where I am today to my parents and my dear friends, Marishka and Couper. You influenced my life the most, helping me to become the person I am today. Mom, you are my "soulmate", my little hummingbird. You are the most warm-hearted, understanding, outgoing, hard-working and passionate person I know. Dad, you have always kept me on track and have always believed in me. I can't count all the times I looked into your eyes and read, "Allison, you are just great" or "Allison, I expected more from you". You have listened to my woes and you always say, "Well, if there's anything I can help with…". I love you so much and it makes me so happy that we have become closer and closer over the years (I think mom's jealous). Marishka, you are quite possibly the most amazing person. No one would believe the hardships you have overcome if they heard all that you have accomplished. I admire your tenacity and adore your lifelong friendship. Couper, I think you know how my life changed because of you. Since we were six our experiences together included earthquakes, Tiger, making piñiatas, rattlesnakes, crushes, babysitting, camping, watermelon-eating contests, limeade sales, changing schools, boyfriends (and their problems), sewing, college, etc. Some of my best memories came from you. Everyone who knows me, knows you, because the devastation of your accident was not only the catalyst to make a lot of changes in my life in the past, but, is also an influence on the way I see life today, so, I think it's fair to say that we are still experiencing things together.

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Last, but not least, I would like to thank my fiancé, Trent. Three words: loving, supportive, massages. He was always understanding when I couldn't spend time with him because I was up in the lab counting 20,000 RBC's/day or working on my thesis. When we did get to hang out, he didn't mind when my version of "spending time together" entailed us going to Barnes and Noble and working together. It definitely helps that he, himself, is so driven. Did I mention that he gives massages?

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1. INTRODUCTION

Babesia species are taxonomically classified in the phylum Apicomplexa, class Piroplasmea, and order Piroplasmida (Levine, 1971). The apical complex organelle (rhoptries, etc.) places the parasite as an Apicomplexan while the pear-shaped intraerythrocytic form of the parasite characterizes it as a piroplasm. *Babesia* species are divided into two groups based on merozoite size. The small *Babesia* are1-2.5 μm and include *Babesia gibsoni*, *Babesia microti*, and *Babesia divergens*. The large *Babesia* species are 2.5- 5.0 μm and include *Babesia bigemina*, *Babesia caballi*, and *Babesia canis*. Based on 18s ribosomal RNA gene sequences, the large and small *Babesia* species do not fall into two corresponding phylogenetic groups (Persing et al., 1995).

Babesia is capable of parasitizing many mammalian species, including canine, bovine, equine, and human. *Babesia* species that cause bovine babesiosis and equine piroplasmosis are of economic importance, supporting the need for chemotherapeutic agents in addition to vaccines. *Babesia equi* and *B. caballi* have a worldwide distribution and are the cause of equine piroplasmosis. Bovine babesiosis, also with worldwide distribution, can be caused by *B. bigemina*, *Babesia major*, *Babesia bovis*, or *B. divergens* (Levine, 1985).

Both *B. equi* and *B. caballi* can be transmitted by *Boophilus*, *Hyalomma*, *Dermacentor*, and *Rhipicephalus* species of tick (Levine, 1985; Friedhoff, 1988). Equine

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This thesis follows the format of the International Journal for Parasitology.

piroplasmosis is characterized by anemia, icterus and fever and, if the horse does not die, a carrier state will be established. The carrier state is of specific importance to race horses because the parasites cause a reduction in the oxygen-carrying capacity of the blood (Hailat et al., 1997; Olivier et al., 1997). *Babesia equi* and *B. caballi* infections also have the potential to keep horses from being imported and exported, which is of major importance to equestrian events such as those that take place at the Olympics (Friedhoff et al., 1990). In the United States, the infection state is determined serologically by the complement fixation test. However, this method of detection may not detect new or acute infections because the equine host may not have had time to mount an immune response. It is critical to produce a vaccine against equine babesiosis that will prevent other equine hosts from being infected. If the equine host becomes infected, treatment depends on the species of *Babesia*. *Babesia caballi* can be cleared with imidocarb, however there is not an effective treatment to clear *B. equi* infections and the dosage of imidocarb that would be required for *B. equi* is close to the LD50 (Adams, 1981; Kuttler, 1981; Kuttler et al., 1987).

Bovine babesiosis can be caused by a number of *Babesia* species, including *B. bigemina, B. bovis, B. divergens*, and *B. major* (Levine, 1985; Mehlhorn and Kakoma, 1994). For historical and pathogenic reasons, the most economically important causes of bovine babesiosis are *B. bigemina*, *B. bovis*, and *B. divergens* (Smith and Kilborne, 1893; Dolman, 1969; Levine, 1985; Mehlhorn and Kakoma, 1994; Bowers, 2000). *Babesia bigemina* and *B. bovis* are said to have been the cause of death of over 15,000 head of cattle in the 1860's after seemingly healthy cattle were transported to Illinois and

Indiana from Texas. During this time, mortality rates approached 90% (Smith and Kilborne, 1893). It was later estimated by the United States Congress that the economic loss associated with the babesiosis outbreak was approximately $130.5 million (equivalent to billions of dollars today) (Dolman, 1969; Bowers, 2000). Besides loss of cattle head, the industry suffered from the severe weight loss and decreased milk production associated with the disease. The cattle fever tick eradication program was put into action years later, clearing the vector ticks for bovine babesiosis, *Boophilus annulatus* and *Boophilus microplus,* from the United States (Bowers, 2000; Marquart, 2000; Burkot and Graves, 2000). However, the tick is endemic in Mexico causing loss of livestock and propagating the number of infected ticks. Texas is still at risk of becoming reinfected as stray and smuggled animals from Mexico cross the border (Bowers, 2000). In fact, 50% of the cattle and 20% of the equids that have been seized during smuggling attempts into the United States were infested with cattle fever ticks (Bowers, 2000).

Babesia bovis, *B. bigemina*, and *B. divergens* are the most economically important causes of bovine babesiosis. *Babesia bigemina* is geographically limited to the tropics and subtropics of Africa, Australia, Europe, the Middle East, Central and South America, and Mexico by the geographic distribution of *Boophilus* ticks (Levine, 1985; Mehlhorn and Kakoma, 1994). *Babesia bovis* has the same geographic distribution as *B. bigemina* and is vectored by *Boophilus* spp. ticks, however, it is also distributed throughout some European countries, where it is vectored by *Ixodes ricinus* (Burkot and Graves, 2000). *Babesia divergens* is limited in geographic distribution to northern Europe and the United Kingdom by the range of the tick vector, *I. ricinus* (Burkot and

Graves, 2000). In addition to being a cause of bovine babesiosis, *B. divergens* is the primary cause of human babesiosis in Europe (discussed below). *Babesia bigemina, B. bovis,* and *B. divergens* are usually highly pathogenic in bovine adults but not in calves and *B. bovis* is generally more pathogenic than *B. bigemina* and *B. divergens* (Levine, 1985; Mehlhorn and Kakoma, 1994). The pathogenesis caused by *B. bigemina*, *B. bovis*, and *B. divergens* is similar except bovine hosts infected with *B. bovis* may incur lesions of the cerebrum and cerebellum, where capillaries are swollen with infected erythrocytes (Mehlhorn and Kakoma, 1994). Generally, acute cases of bovine babesiosis will be associated with a high temperature (106-108ºF), lethargy, and hemoglobinuria, and might result in death if the bovine host is not treated (Levine, 1985). Chronic cases of bovine babesiosis generally do not present a high temperature or hemoglobinuria but are lethargic and unwilling to eat, resulting in reduced milk production and poor weight gain (Gray and Harte, 1985; Levine, 1985; Gorenflot et al., 1998). Cattle that survive will be premunized by a latent infection, which will last for the life of the bovine host if it is infected by *B. bigemina*. If the bovine host is infected by *B. bovis* or *B. divergens*, premunization will last no more than two years without a boost in immunity by re-infection (Levine, 1985).

Babesia bovis, B. bigemina, and *B. divergens* can be treated with imidocarb, but the goal of treatment is to reduce parasitemia and pathogenesis, leaving the bovine host to develop a carrier state (Mehlhorn and Kakoma, 1994). Vaccines have been designed to incorporate the fact that young cattle in endemic regions display a level of protection against infection with the *Babesia* species of that region. It is uncertain what factor(s)

influence the protection seen in calves. A relationship has been shown between age-related immunity and the release of Th1 cytokines (IL12, IFN) in addition to natural killer cells and nitric oxide synthase mRNA expression in the spleen (Goff et al., 2001; Goff et al., 2002; Goff et al., 2003). When the calves are infected, they are usually protected from developing signs of disease. The calves become carriers after exposure, premunized against future infection. Cattle older than 6 months of age are not protected (Christensson, 1989). Vaccines that premunize young cattle by inoculation with live *Babesia* parasites protect against natural infection by mounting a strong and rapid immune response to prevent disease (Mahoney, 1977). However, problems associated with this vaccine design will be discussed below.

Other important species of *Babesia* include *Babesia canis* and *B. gibsoni*, which are the primary species that infect domestic dogs and wild canids. *Babesia canis* and *B. gibsoni* are found worldwide and can be transmitted by *Rhipicephalus sanguineus* or *Dermacentor reticulatus* ticks (Levine, 1985). *Babesia canis* pathogenesis may involve the circulatory and respiratory systems, eyes, and central nervous system. When the central nervous system is involved, the signs might be confused with rabies (Levine, 1985). Dogs that are recovering from *B. canis* will continue to suffer from erythrocyte loss despite declining parasitemias, which does not occur among other *Babesia* species. *Babesia gibsoni* is highly pathogenic in dogs, causing marked anemia, fever, hepatosplenomegaly, constipation, and, contrary to *B. canis*, hemoglobinuria. Another difference between *B. gibsoni* and *B. canis* is that the pathogenicity of the latter varies depending on subspecies (Levine, 1985; Zahler et al., 1998). Importation of dogs into

B. gibsoni-endemic regions carries serious risk, with infected dogs dying within four weeks (Levine, 1985). Treatments are not as effective against *B. gibsoni* as they are against *B. canis,* however, dogs that have recovered from pathogenesis caused by *B. canis* or *B. gibsoni* will remain in a state of premunition in endemic areas. In the absence of re-infection, protection only lasts up to a year (Levine, 1985).

The first documented case of human babesiosis occurred in Europe in a Yugoslavian man in 1957 (Skrabalo and Deanovic, 1957). Since then, more than 30 cases have been reported in Europe and a number of cases have been reported in China, Egypt, Mexico, South Africa, and Taiwan. Hundreds of cases have been reported in the United States (Osorno et al., 1976; Li and Meng, 1984; Michael et al., 1987; Bush et al., 1990; Shih et al., 1997). The clinical presentation of human babesiosis can range from a silent infection to a fulminating disease that is comparable to malaria, resulting in severe hemolysis. Splenectomized and other immune-suppressed individuals have more severe clinical signs, sometimes resulting in death. Human babesiosis in Europe is most commonly caused by *B. divergens*, with cases coinciding with the seasonal activity of *I. ricinis* and close proximity to cattle ranches, whereas the primary cause of human babesiosis in North America is *B. microti,* which is vectored by *Ixodes scapularis* (syn. *Ixodes dammini* (Oliver et al., 1993)). There have been a few cases of human babesiosis caused by *B. microti* in Europe, but the tick vector, *Ixodes trianguliceps*, does not feed on humans under normal circumstances so transmission is therefore unlikely (Telford et al., 1993; Gorenflot et al., 1998). There have also been a small number of human babesiosis cases caused by *Babesia* organisms referred to as WA1, CA1, and MO1,

which have slight phylogenetic differences from other characterized *Babesia* species (Quick et al., 1993; Thomford et al., 1994; Persing et al., 1995; Herwaldt et al., 1996).

In the United States, more than 300 cases of human babesiosis have been diagnosed since 1969 and within the last decade the frequency of diagnosed cases has increased (Kjemtrup and Conrad, 2000). The rise in the number of diagnosed cases might be attributed to a number of factors, which may include increased awareness in the medical community, more individuals participating in outdoor activities, and urban overlap with environments endemic for the reservoir host, *Peromyscus leucopus* (white footed mouse), and the vector tick, *I. scapularis* (Healy et al., 1976; Etkind et al., 1980; Spielman et al., 1981; Piesman et al., 1987; Gorenflot et al., 1998; Kjemtrup and Conrad, 2000). One reason for increased awareness among medical professionals is the fact that *B. microti* can be co-transmitted with *Borrelia burgdorferi*, the causative agent of Lyme disease, by *I. scapularis* (Hofmeister et al., 1998).

Babesia microti, which produces flu-like symptoms in addition to hemolytic anemia, can be life threatening in immune-compromised patients. Individuals who are not immune compromised may still experience persistent parasitemia and fatigue, despite treatment. There is also concern associated with transmission of the parasite through blood transfusion or transplacental transmission, in addition to organ transplant acquired cases (Jacoby et al., 1980; Grabowski et al., 1982; Marcus et al., 1982; Smith et al., 1986; Esernio-Jenssen et al., 1987; Mintz et al., 1991; Slovut et al., 1996; New et al., 1997; Dobroszycki et al., 1999; Lux et al., 2000).

1.1. Ixodes life cycle

Babesia microti is transmitted through the saliva of the blacklegged deer tick, *I. scapularis*, which has three stages of development: larvae, nymph, and adult. Each stage requires a blood meal to undergo the morphological changes into the next stage. The adult tick will feed during the fall and spring on the white tailed deer, *Odocoileus virginianus*, which does not carry *B. microti* infection. In the spring, they will lay eggs, which will hatch into larvae in the summer. The larvae feed preferentially on the white-footed mouse, *P. leucopus*, and to a lesser extent, the meadow vole, *Microtus pennsylvanicus*, during August and September (Healy et al., 1976). During this stage, the tick can pick up the parasite from an infected rodent. In 1980 Etkind et al. found *B. microti* endemic in the New England white-footed mouse, with infection rates of 40-60% (Etkind et al., 1980; Spielman et al., 1981). *Ixodes scapularis* larvae over-winter, molt to the nymph stage, and then feed indiscriminately from May to July. During the nymph stage, the *Ixodid* tick either has another opportunity to pick up the parasite when feeding on a rodent, or, a nymph previously infected at the larval stage can transmit the parasite to a rodent or human (Piesman et al., 1986). The nymph will then molt to the adult stage in the fall and feed preferentially on the white tailed deer. Eggs are laid in the spring, thus completing the two-year life cycle.

1.2. Life cycle of Babesia species in Ixodid ticks

The life cycles of all species of *Babesia* are relatively similar except that *B. gibsoni*, *B. equi,* and *B. microti* do not undergo transovarial transmission through the vector tick

and the latter two have a pre-erythrocytic schizont stage in lymphocytes (Moltman et al., 1983; Mehlhorn et al., 1986). It is presumed that *Babesia gibsoni* also infect lymphocytes during the schizont stage. *Babesia equi*, *B. microti*, and *B. gibsoni* also undergo simultaneous division resulting in tetrad formations of the merozoites, whereas *Babesia sensu stricto* divide into paired merozoites.

The *Ixodid* tick will pick up erythrocytes infected with *Babesia* during a blood meal. In the tick gut, some of the intraerythrocytic parasites will develop into gametes, which will fuse and become a zygote (Telford et al., 1993). The zygote then becomes a motile kinete that migrates through the gut and hemolymph to penetrate a variety of organs, including fat body cells, nephrocytes, ovary and salivary glands. *Babesia sensu stricto* species will undergo transovarial, and sometimes, transstadial transmission, whereas *Babesia sensu lato* species (*B. equi, B. microti,* and *B. gibsoni*) only undergo transstadial tick transmission (Mehlhorn and Schein, 1984; Sonenshine, 1991). Transovarial transmission is the process of the kinetes undergoing asexual reproduction in the ovary of the adult female tick, infecting the developing eggs, and producing infected larvae after hatching. Transstadial transmission is signaled once the tick larvae or nymph begins feeding on a vertebrate host. The kinetes migrate to the fat body cells, nephrocytes, and salivary glands where they undergo asexual reproduction (Mehlhorn and Schein, 1984; Telford et al., 1993).

Within days of the infected tick attaching to a host, the kinetes migrate to the salivary glands to produce sporozoites by sporogony (Mahoney and Mirre, 1977; Mehlhorn and Schein, 1984). The sporozoites are inoculated into the host with the aid of

anti-inflammatory and immunosuppressive chemical agents in the tick saliva, which increase the efficiency of transmission (Ribeiro, 1987). The sporozoites then invade erythrocytes and undergo merogony to produce two to four merozoites (Rudzinska et al., 1976). The merozoites exit the cell and infect more erythrocytes, where they either differentiate into trophozoites, which replicate by budding, producing more merozoites. Alternatively, the merozoites may enlarge and become gametocytes, which will only differentiate if they make it back to the tick gut (Rudzinska et al., 1976; Rudzinska et al., 1979; Rudzinska et al., 1981; Mehlhorn and Schein, 1984; Levine, 1985).

1.3. Morphology

In Giemsa-stained blood smears, *B. microti* parasites appear as small (1-3 μm) round or oval rings with darkly staining nucleic material to one side of the ring. Small red dots may also be seen in with nucleic material. The vacuole within the ring stains light blue to white. As the parasitemia increases the parasites take on a wide-ranged morphology including larger sized oval rings, multiply infected erythrocytes, pear-shaped (piriform), and maltese cross (tetrad) forms.

1.4. Immune response and requirements

In the United States, cases of human babesiosis attributed to *B. microti* range in severity from asymptomatic to highly pathogenic and fatal. The degree of parasitosis is directly related to the efficiency of the host immune response (Parham, 2000). Most

severe cases are observed in individuals with an immune deficiency, such as previous splenectomy or acquired immune deficiency syndrome (AIDS) (Markell et al., 1999).

Once *Babesia* species invade the physical barrier of the host, the host's innate immunity may mount a futile inflammatory response, in addition to producing carbohydrate binding proteins (to fight the invading microorganisms) (Parham, 2000). When *Babesia* parasites reach the vascular system, the sporozoites circulate freely in the plasma. At this point, the parasites are open to attack by the humoral immune system. Immunoglobulin G (IgG) antibodies can bind to the parasites and label them for elimination by lymphocytes. However, this stage in the parasite cycle is limited. Once the parasites enter the erythrocytes, the humoral response is ineffective against the now intracellular parasite. In fact, Echaide et al. (1998) described the expression of a *B. bigemina*-originating protein on the surface of erythrocytes that helps bind IgM, suggesting that IgM binding might play a role in parasite growth and survival. This was supported by earlier research that showed that IgM suppressed mice were resistant to *B. microti* infection (Rosenberg, 1979). At this stage of the infection, the most effective immune response to inhibit *Babesia* is CD4+ T helper cells and the subsequent release of IFN-γ (Ikuo et al., 1994; Shimada et al., 1996).

Several studies have emphasized the importance of Th1 cytokines in host defense mechanisms against infection caused by various microbial pathogens (Mehlhorn and Schein, 1998). One of the main problems associated with intracellular parasitic infections is that a host Th2 response is stimulated, rather than Th1, which exacerbates the parasite infection (Keene et al., 1990). The presence of CD4+ T cells and IFNγ, the

release of which is mediated by a Th1 immune response, greatly influences the ability of the host to clear parasitemia. In fact, mice are more susceptible to infection with *B. microti* when they are depleted of CD4+ helper T cells, and, consequently, IFN. Moreover, the ability of mice to fight infection with *B. microti* and *B. rodhaini* is unaffected when they are depleted of CD8+ helper T cells (Igarashi et al., 1994; Shimada et al., 1996). Natural Killer (NK) cells and macrophages may also play a role in fighting babesiosis (Aguilar-Delfin et al., 2003). NK cells and macrophages, in addition to T cells and B cells, make up the cell populations within the spleen, an organ critical to fighting babesiosis (Mehlhorn and Kakoma, 1994; Aguilar-Delfin et al., 2003). Ultimately, the immune system of an animal or individual with babesiosis primarily requires not only a Th1 response, in order to activate a cytotoxic T-cell response, but also a Th2 response in order to activate some protective immunity against re-infection or re-establishment of disease.

1.5. Chemotherapy

It is important to note that animals may go into a chronic carrier-state after being infected by a *Babesia* sp., acting as a reservoir of parasites for tick vectors to pass on to other animals (Levine, 1985). When naïve animals are brought into these areas, the mortality rate can be as high as 90% (Dolman, 1969; Markell et al., 1999). In a study of humans infected with *B. microti* by Krause et al., it was found that a silent *Babesia* infection could persist for months to years if the individuals are not treated with anti-*Babesia* chemotherapeutic agents (Krause et al., 1998). They also found that individuals

who receive treatment with clindamycin and quinine might still suffer from a persistent infection, re-establishment of parasitemia, or side effects from the drugs. An alternative is the combination of atovaquone and azithromycin, also associated with adverse side effects but not as commonly. Chemotherapeutic agents do not protect against future infection and the parasite may go dormant. Consequently, an animal under stress may experience recrudescence of parasitemia. This highlights the importance of developing a vaccine that elicits long-term protection. For now, chemotherapeutic agents should still be used because they are usually effective in lowering parasitemia and the clinical signs of babesiosis, with the exception of individuals who are immune suppressed (Jacoby et al., 1980; Smith et al., 1986; Krause et al., 1998).

1.6. Vaccine designs

The frequency of human infections with *B. microti* is not high enough to invest in large-scale vaccine production. On the other hand, working with *B. microti* allows the use of mice rather than large mammals for vaccine trials all the while gaining knowledge for vaccine designs that may work for more economically important *Babesia* species.

Various *Babesia* vaccines were examined in the past for immunization against *B. canis*, *B. bovis, B. bigemina*, or *B. equi*, but not *B. microti* (Callow, 1971; Singh et al., 1981; Wright, 1991; Bock et al., 1992; Kung'u et al., 1992; Echaide et al., 1993; Lawrence et al., 1993; Goodger et al., 1997; Ruef et al., 1997; Schetters et al., 1997; Edelhofer et al., 1998; Kumar et al., 2002b; Pipano et al., 2002). Vaccine designs have included live virulent or attenuated parasites, killed merozoites, soluble parasite

antigens, and recombinant proteins. The first *Babesia* vaccine produced was developed for *B. bovis* and employed an attenuated strain that was developed in splenectomized calves (Callow, 1971; Callow, 1979; Callow et al. 1997). The success of the *B. bovis* attenuated vaccines encouraged the development of attenuated vaccines for *B. bigemina* and *B. divergens* (Echaide et al., 1993; Edelhofer et al., 1998). Immunization of *B. bigemina* has incorporated the use of live attenuated or virulent organisms (Pipano et al., 2002). Usually, the cattle are immunized when they are young because cattle younger than 6 months display protection against developing signs of disease. At this time, the reason for this protection is unknown. It has been suggested that antibodies from the colostrum of an exposed mother confer protection in the calf. However, Christensson (1987) showed that calves that do not receive colostrum still display the same level of protection. Furthermore, Levy et al. (1982) demonstrated that the factor(s) responsible for resistance are found in the plasma of young calves. There is more risk involved in immunizing older animals, which don't have the protection that younger cattle have, so chemotherapy may be required to control the infection. This method should only be used in endemic areas because it is only dependable for keeping the cattle from developing clinical signs of disease, not infection. Therefore, the vaccinated animals serve as a reservoir for infection.

Immunization with live *Babesia* organisms serves to benefit endemic regions such as within Australia and Africa. There have even been some signs of cross-strain immunity. In an experiment testing the Australian *B. bovis* vaccine on three calves in Malawi, all three calves were given full protection against challenge with an African strain of

B. bovis, despite differences between the Australian and African strains (Lawrence et al., 1993). Regardless of the benefits of using this vaccine design, cattle that are not continuously reinfected can clear infection, which results in dissolution of protection from disease. Pipano et al. (2002) conducted a study of 42 Friesian cows that had been previously vaccinated two times. They found that only 11 of 22 cows had persistent *B. bovis* infection and only two of 20 cows had persistent *B. bigemina* infection. Besides lack of reinfection, strain-specific resistance can result in cattle developing acute disease to heterologous strains (Callow et al., 1967; Bock et al., 1995). Lack of reinfection and strain variation limits the effectiveness of immunization with live *Babesia* organisms and places boundaries on the movement of cattle, despite "protection". The biggest economic drawback to live vaccines is the limitation placed on the importation and exportation of cattle.

Another problem associated with the live or attenuated vaccine design is the difficulty in producing enough parasites on a large enough scale from splenectomized calves. In Australia, vaccine producers have had to struggle to produce enough parasites to satisfy the demand for their attenuated live trivalent tick fever vaccine containing *B. bovis*, *B. bigemina,* and *Anaplasma centrale* (Standfast, 2003). Moreover, despite the effectiveness of live attenuated vaccines, their use puts the vaccinated animal at risk for contracting other enzootic agents, such as bovine leucosis virus, when they receive the infected blood for the attenuated vaccine (Wright, 1991). Furthermore, attenuated vaccines suffer a short shelf life (Wright, 1991). These shortfalls have led to research of

alternatives such as killed vaccines, recombinant vaccines, and vaccines derived from the soluble antigens of in vitro cultures.

A killed vaccine against equine babesiosis caused by *B. equi* was designed using killed merozoites (Kumar et al., 2002b). The vaccine was prepared from *B. equi* infected blood containing a lysate of $2x10^{10}$ infected erythrocytes per dose combined with the adjuvant Quil A, followed by a booster inoculation. The vaccine was tested on donkeys, showing protection among the vaccinated donkeys whereas the control donkeys died from challenge. Also, the parasitemia in the vaccinated group cleared and was not reestablished after splenectomy. These results are probably not due to the merozoite lysate alone, as Singh et al. carried out a similar experiment reporting that the immunized donkeys remained carriers and that parasitemia reappeared after immune suppression (Singh et al., 1981). A major difference between the two reports was the use of the adjuvant Quil A. Quil A is known to evoke a strong Th1 immune response, which is why it is commonly used as an adjuvant for some viral vaccines. Also, there may have been strain differences corresponding to less or more virulence between the strains. With the encouraging results of the study conducted by Kumar et al. (2002b), it is surprising that there have not been any further developments reported.

Soluble parasite antigens (SPA) have been used for a number of *Babesia* vaccines in order to stimulate protection without resulting in carrier status (Goodger et al., 1987; Goodger et al., 1992; Montenegro-James et al., 1992; Schetters et al., 1997; Schetters et al., 2001). The antigens of SPA vaccines may be exogenous, derived from the supernatant of *in vitro Babesia*-infected erythrocyte cultures, or endogenous, derived

from *Babesia*-infected erythrocytes (*in vitro* or *in vivo*) that are sonically-disrupted. The *B. canis* SPA vaccine, used in Europe under the name 'Pirodog', is made up of exogenous antigens (Moreau et al., 1988). The vaccine limited the level of parasitemia, the decline in packed cell volume (PCV), anemia, and splenomegaly associated with infection (Schetters et al., 1997). However, *B. canis* SPA vaccine is not effective in protecting against heterologous challenge (Schetters et al., 1995). As a result, Schetters et al. (2001) tested the effectiveness of a *B. canis canis*-*B. canis rossi* SPA vaccine against heterologous challenge, inducing an immune response that resulted in the parasitemia in the vaccinates lower than in the controls.

A culture-derived endogenous *B. bovis*- *B. bigemina* SPA vaccine was tested in Venezuela where bovine babesiosis is endemic and imported cattle suffer significant losses (Montenegro-James et al., 1992). The vaccine reduced the incidence of clinical disease and prevented death, however, the level of protection was not as high as with live vaccines. Alternatively, an endogenous (as opposed to exogenous) SPA vaccine against *B. bovis* was derived from a fraction of infected erythrocytes collected from infected cattle (Goodger et al., 1987; Goodger et al., 1992). The vaccinated cows had a lower mean daily parasitemia and survived homologous challenge, whereas the controls had a higher daily parasitemia and did not survive challenge. Though *B. bovis* SPA vaccines are protective against homologous challenge, results of heterologous challenge have been mixed. Montenegro-James and Ristic (1985) elicited protection against heterologous challenge using a SPA *B. bovis* vaccine. In contrast, Timms et al. (1983) showed that SPA do not effectively protect against heterologous *B. bovis* challenge. SPA

vaccines are safer, more stable, and easier to develop than live vaccines. However, the protection evoked by the soluble parasite antigen does not surpass that of live vaccines and live vaccines appear to be more dependable at this time.

Recombinant vaccines have also been explored for a number of *Babesia* species, targeting fusion proteins, lipoproteins, and surface proteins. Fusion proteins are genetically engineered to include two or more proteins or peptides. Wright et al. (1992) selected two proteins, glutathione-S-transferase -12D3 and -11C5 (GST-12D3 and GST-11C5, respectively), after vaccinating with fractionated crude extracts of *B. bovis* parasites and purifying the protective fractions by affinity chromatography with monoclonal antibodies. When GST-12D3 and GST-11C5 were combined and tested against *B. bovis,* they elicited strong protection, but not as strong as the live vaccine.

Goodger et al. (1992) targeted *B. bovis* lipoprotein as a vaccine candidate because a previous vaccine trial carried out by the author suggested lipoprotein involvement in antibody production (Goodger et al., 1987). In order to determine if lipoprotein elicits protective antibodies against *B. bovis*, calves were injected with lipoprotein (precipitated by dextran sulphate) and then serologically tested for lipoprotein-specific antibodies and monitored by blood smear. The vaccinated calves showed a delayed and decreased parasitemia compared to controls (Goodger et al., 1992). The vaccinated group also differed from controls by producing an antibody response that included high levels of IgG1 (Th2) and IgG2 (Th1), whereas the controls remained close to baseline (Goodger et al., 1992). However, there was not a control for the dextran sulphate, administered in

conjunction with the lipoprotein. As a matter of fact, Diamantstein et al. (1973) showed that dextran sulphate elicits high antibody titers.

The major surface antigens associated with the parasite apical complex, such as the rhoptry-associated proteins, have been targeted because they play a role in host cell invasion. Hypothetically, if the host immune system is directed to have a strong inhibitory response to a surface protein of the apical complex, host cell invasion will be inhibited and the parasite life cycle cannot progress. Ruef et al. (1997) targeted the rhoptry-associated proteins for a recombinant vaccine because they are part of the apical complex of *Babesia* parasites, which is involved in host erythrocyte invasion (Perkins, 1992). Rhoptry-associated protein 1 (RAP-1) was used to immunize cattle against *B. bigemina*, inducing a Th1 cytokine response, but neither the specific RAP-1 antibodies nor the level of Th1 cytokines had a direct correlation with the degree of protection against challenge (Ruef et al., 1997). Another downside to recombinant vaccines is that the protection is short-lived, requiring continual boosts. Further, long-term studies of boosted recombinant proteins have not been carried out.

The current vaccines have had limited effectiveness and while they may protect against disease, they do not give full protection against future infection. Previous anti-*Babesia* vaccines, such as live-attenuated or killed parasites, are usually strain-specific and may lose their effectiveness over time because of slight natural field-strain variation. Field investigations in Australia uncovered a nine-fold increase in the failure of the live *B. bovis* vaccine to protect cattle from 1985 to 1990 due to changes in the field-strain of *B. bovis* (Bock et al., 1992). At this point, it is still clear that the live vaccine is the most

effective currently available vaccine, however, future vaccine designs should address the need for a vaccine that is not based on maintaining a carrier status and is not strain-specific. Such a vaccine would be more beneficial to cattle and equine industries, allowing trade and importation of livestock in and out of endemic areas to occur more easily.

1.7. Future focus for vaccine development

Current strategies for *Babesia* vaccines should incorporate a Th1 response in addition to a Th2 immune response. A Th1 response is critical for organisms that are intracellular because the Th2 response does not affect any intracellular stages. Antigen presenting is an important determinant of the type of helper T cell response (Parham, 2000). In order to drive Th1 differentiation, the antigen presenting cells must be associated with the release of interleukin-12, which are produced during the beginning stages of infection (Avila and Calderon, 1993). The role of antigen presenting cells in the Th2 immune response is to present the antigen to B cells. Antigen presentation to B cells promotes differentiation into plasma cells or memory cells, which will produce antibody to the extracellular stages of the *Babesia* species (Parham, 2000).

Protection in other parasitic diseases has also shown the need for a Th1 immune response. Many chemotherapeutic and vaccine targets have effectively stimulated a Th1 immune response (Doolan et al, 1996; Das et al., 2001; Wang et al., 2004). One avenue that has been targeted is the cathepsin L-like cysteine proteases. Research regarding the

use of cysteine proteases in vaccines, for the most part, has shown a shift towards the Th1 response after parasitic challenge.

1.8. Cysteine protease as a target for vaccine development

Cysteine protease inhibitors have combated a number of parasitic infections, demonstrating that cysteine proteases are critical for survival and proliferation of parasites (d'Oliviera et al., 1997; Engel et al., 1998; Das et al., 2001). Alexander et al. (1998) found that inhibition of *Leishmania mexicana* cathepsin L-like proteases resulted in a substantial reduction in virulence and a shift from a Th-2 to a Th-1 immune response. Additionally, cysteine protease inhibitors have been shown to inhibit erythrocyte invasion by *Plasmodium falciparum*, inhibit tissue invasion and necrosis by *Entamoeba histolytica*, and block invasion of *Theileria parva* sporozoites into lymphocytes (Rosenthal, 1989; De Meester et al., 1990; Syfrig et al., 1998; Greenbaum et al., 2002). The replication and life cycle growth of parasitic protozoans and helminths can be inhibited, without toxicity, by selectively inhibiting the cysteine protease of the parasite (McKerrow, 1999).

Cysteine proteases have vital roles in mammalian cellular turnover and apoptosis, but they also play a role in the life cycle of many parasites (Mathews et al., 2000; Sjid and McKerrow, 2002). The cysteine proteases of parasites, like mammals, have catabolic and protein processing functions. However, unlike mammalian cysteine proteases, cysteine proteases of parasites may play a role in immune evasion, excystment/ encystment, cell invasion, and, in a few cases, tissue invasion (Sjid and McKerrow,

2002). Accordingly, the actions of the cysteine protease(s) may differ among different genera of parasites. The cathepsin L-like cysteine proteases can be differentiated from other classes of cysteine proteases by conserved critical residues (Fig.1., p. 33), the presence of cysteine-histidine-asparagine members of the catalytic triad, and an ERFNIN-like motif in the pre-pro region. However, Sjiid and McKerrow (2002) have found that there is still little variance between the cysteine proteases of parasites. Cysteine proteases are not only an attractive target for vaccine and chemotherapy designs because they play important roles in the life cycle of the parasite, but also because they are known to elicit antibodies during natural infection. Inhibition of the life cycles of *B. equi* and species of *Plasmodium, Leishmania, Trypanosoma, Fasciola,* and *Entamoeba* has been elicited by targeting the cysteine protease. Therefore, the cysteine proteases of *B. microti* may be a good target for vaccine and chemotherapy development (Wijffels, 1994; Mulcahy and Dalton, 2001).

1.9. Babesia cysteine proteases

Holman et al. (2002) showed *in vitro* that inhibition of the *B. equi* cysteine protease inhibits propagation of the parasites. Two cysteine protease inhibitors, E64 and E64d, were used independently to help elucidate the activity of the *B. equi* cysteine protease. In the experiment, only E64d, which is membrane permeable, brought about parasitic inhibition. The cysteine protease inhibitor E64, which is not membrane permeable, did not differ significantly from the controls. These results suggested that the cysteine

protease activity is associated with intraerythrocytic functions or hemolysis associated with merozoites leaving the erythrocyte (Holman et al., 2002).

Babesia microti encodes for two cathepsin L-like cysteine proteases, which play a major role in life-cycle growth and cell invasion (Mahmoud et al., 2002).

1.10. Plasmodium cysteine proteases

Plasmodium falciparum expresses three papain-family cysteine proteases, known as falcipain, that are active during the trophozoite and ring stages. At that point in the life cycle, the cysteine proteases degrade host hemoglobin in order to provide amino acids for parasite protein synthesis and growth (Rosenthal, 1989; Gluzman et al., 1994; Francis et al., 1997; Rosenthal et al., 2002). After the cysteine proteases assist in merozoite invasion of erythrocytes and hemoglobin degradation, falcipain-2 assists in late schizont-mediated rupturing of erythrocytes to release more parasites into the blood (by destabilizing the ankyrin-band 3 cytoskeleton of the erythrocytes). Inhibition of the cysteine protease falcipain-2 substantially inhibits the life cycle of *P. falciparum* (Rosenthal et al., 2002).

1.11. Leishmania cysteine proteases

The cathepsin L- like cysteine proteases found in all species of *Leishmania* are required for the parasite life cycle and pathogenicity (Seltzer et al., 1999). *Leishmania mexicana* cysteine protease plays a major role in parasitic invasion of macrophages and intracellular survival of the parasite (Frame et al., 2000). It has also

been shown by deletion of the entire copy of the cysteine protease gene that this enzyme is responsible for suppressing the host Th1 immune response to *L. mexicana* (Buxbaum et al., 2003). Further, Ilg et al. (1994) determined that immunization with *L. mexicana* recombinant cysteine proteinase stimulates the development of a Th1 immune response.

Leishmania donovani virulence also depends on the presence of cysteine proteases. When the cysteine protease of *L. donovani* was inhibited by a cathepsin B inhibitor, the lifecycle was disrupted and parasitemia cleared (Somanna et al., 2002).

The cysteine protease activity of *Leishmania* species assists in the ability of the parasite to infect and replicate in the host and appears to correlate with the degradation of host major histocompatability complexes (MHC), which in turn cannot present antigens to B cells or T cells, inhibiting the host immune response (Descoteaux, 1998; Courret et al., 2001). Consequently, inhibition of the cysteine protease genes by homologous recombination results in loss of *Leishmania* pathogenicity (Selzer et al., 1999). Therefore, the cysteine protease of *Leishmania* species has been seen as an attractive target for vaccine development. BALB/c mice vaccinated with purified cysteine protease, exhibited protection against *L. major* infection following parasite challenge (Rafati et al., 2000, 2001).

1.12. Trypanosoma cysteine proteases

Leishmania and *Trypanosoma* species have similar cysteine proteases that are characterized by an unusual 100 amino acid C-terminal sequence. Mutant *Leishmania* and *Trypanosoma* species lacking the cysteine protease have a reduced infectivity of

macrophages in vitro and in BALB/c mice (Alves et al., 2001). Cruzipain, the major cysteine protease of *Trypanosoma cruzi*, has vasodilatory activity, which is brought about by cruzipain release of kinins. The kinins induce plasma leakage, which allows *T. cruzi* to escape from the blood vessel into nearby tissues (Mulcahy and Dalton, 2001).

A DNA vaccine encoding cruzipain induced protective Th1 cytokines (IFN) and cytotoxic T lymphocytes in a mouse model (Schnapp et al., 2002a). Further, the authors showed that vaccination of mice with the DNA vaccine encoding cruzipain evoked a significant level of protection against *T. cruzi* and Th1 cytokines including IFN and IL-12 (Schnapp et al., 2002b).

1.13. Babesia vaccine design

Babesia species like other parasitic protozoans, such as *Plasmodium, Leishmania, Trypanosoma,* and *Entamoeba,* and parasitic helminths, such as *Fasciola* species, encode similar cathepsin L-like cysteine protease(s), which may play a key role in immune evasion, virulence, and invasion into tissue as well as the cell (Rosenthal, 1989; Keene et al., 1990; Avila and Calderon, 1993; Gluzman et al., 1994; Francis et al., 1997; Alexander et al., 1998; Descoteaux, 1998; Frame et al., 2000; Cordova et al., 2001; Greenbaum et al., 2002; Holman et al., 2002; Mahmoud et al., 2002; Rosenthal et al., 2002; Sjid and McKerrow, 2002). Cysteine proteases have been targeted for vaccines and chemotherapy against *Babesia, Plasmodium, Leishmania, Trypanosoma, Entamoeba,* and *Fasciola* species, resulting in inhibition of the parasite life cycle (Healy et al., 1976; Rosenthal, 1989; Wijffels, 1994; Oliaro and Goldberg, 1995; Engel et al.,

1998; Selzer et al., 1999; Rafati et al., 2000; Rafati et al., 2001; Bangs et al., 2001; Das et al., 2001; Mulcahy and Dalton, 2001; Holman et al., 2002; Rosenthal et al., 2002; Schnapp et al., 2002a-b).

The vaccine designed for this study targets a protein in *B. microti* shown to be critical to *in vitro* replication of *B. equi* (Holman et al., 2002; Mahmoud et al., 2002). *Babesia microti* cysteine protease-1 was selected because, of the two-cysteine proteases identified in *B. microti*, it is most similar to the characterized *B. equi* cysteine protease. Therefore, a vaccine designed with the *B. microti* cysteine protease-1 will make a better model for *B. equi*.

The vaccine design encompasses a prime boost of plasmid DNA with the *B. microti* cysteine protease-1 (Bmcp) insert, followed by two boosts of cysteine protease-1 peptide (CP) or two boosts of plasmid DNA with Bmcp-1 at two-week intervals. It has been shown that the prime-boost strategy can enhance the immunogenicity of the plasmid DNA vaccine (Sedegah et al., 2000; McConkey et al., 2003; Rainczuk et al., 2003; Ramiro et al., 2003; Sedegah et al., 2003; Wang et al., 2004). In a study by Rainczuk et al. (2003), the mice that were boosted with recombinant protein had a significantly higher rate of survival against *Plasmodium chabaudi adami* challenge when compared to the controls, in addition to exhibiting higher IgG2 levels (Th1) and lower IgG1 levels (Th2) than mice that were only primed with the plasmid DNA. The host immune response to plasmid DNA vaccination involves the uptake of DNA by host cells followed by antigen presentation on the cell surface and uptake by antigen presenting cells (APCs). The specific immune response is then determined (Mumper and Ledebur,

2001). It is anticipated that Bmcp will be taken up by the host cells and presented as antigen on the cells, to be taken up by APCs, stimulating primarily a Th1 immune response and secondarily a Th2 response. DNA-based vaccines have the ability to encode antigens to induce both CD4+ and CD8+ T cells and, therefore, the ability to elicit humoral and/or cell-mediated immunity against parasitic infections. A DNA vaccine against *Plasmodium yoelii,* encoding the circumsporozoite protein (CS), elicited high levels of CS-specific antibody and CD8+ T cells, resulting in a protection rate of 68% (Sedegah et al., 1994). Plasmid DNA has been used as a vector of antigen to immunize against numerous parasitic infections, displaying protection against multiple species of *Plasmodium*, *Leishmania, Theileria*, *Trypanosoma,* and *Fasciola* (Sedegah et al., 1994; Xu and Liew, 1995; Doolan et al., 1996; d'Oliveira et al., 1997; Montgomery et al., 1997; Alarcon et al., 1999; Sepulveda et al., 2000; Smooker et al., 2000; Kumar et al., 2002a). Another benefit is that the immune response to DNA vaccines is usually long lasting (Alarcon et al., 1999).

The vaccine design in this study utilizes intramuscular (i.m.) injection for priming with the pDNA with Bmcp-1 insert. It was previously demonstrated by Wolff et al. (1990) that i.m. vaccination with plasmid DNA encoding an inserted protein will result in uptake and expression of the encoded protein by mouse skeletal muscle cells. Also, the plasmid DNA is taken up without integrating into the mouse genomic DNA, existing in the cells in an extrachromosomal form. Wolff et al. (1992) further found that i.m. injection of plasmid DNA also has long-term expression, as shown by the presence of foreign genes in the muscle of mice for at least 19 months following injection. Thus, i.m.

injections of pDNA can be a safe and effective method for expressing and presenting parasite antigens to evoke an immune response by the host (Montgomery et al., 1997).

In this study, the plasmid DNA (pDNA) encoding the cysteine protease will be injected i.m., after which it is anticipated that the Bmcp-1 protein will be expressed extracellularly and taken up by APCs. The APCs will then stimulate an immune response that is aimed towards inhibiting the cysteine protease when the host mouse is challenged by infection with *B. microti*. Targeting the cysteine protease will hypothetically keep the parasite from being able to invade the host erythrocytes, inhibiting the parasite from following a normal life cycle, resulting in a reduced parasitemia. If successful, this vaccine strategy may be applicable to *B. equi*, which has similar cysteine proteases. Successful application would also stimulate interest in determining whether other economically important *Babesia* species encode for cysteine proteases that might be targeted in recombinant vaccines.

1.14. Hypothesis

Vaccination of mice with pDNA with Bmcp1 insert and the gene products will elicit a protective immune response in BALB/c mice against *B. microti* challenge when administered using a prime-boost strategy.

2. MATERIALS AND METHODS

2.1. Preparation of pDNA with Bmcp1 insert and pDNA without insert

The Bmcp1 gene was previously inserted into the entry vector (pENTR-His-*ccd*B; Parr and Ball, 2003). Transduction of the entry vector with the destination vector, pYES-DEST52 (pYD52), resulted in pYD52 with the Bmcp1 gene insert. To ensure proper recombination, the resulting plasmid was transformed into DH5 competent cells. When the transformation was plated on LB plates with 100 g/ml ampicillin (15 h, 37 ºC), only colonies of the DH5 cells with pYD52 with the Bmcp1 insert were capable of growth because the new *att*B recombination sites form a site for ampicillin resistance. If transformation failed, pENTR with the gene insert would not have been capable of growth because it does not have ampicillin resistance and pYD52 would not have been capable of growth because of the intact *ccd*B site that inhibits gyrase, which is necessary for DH5 growth. To reconfirm the presence of the Bmcp1 insert, multiple colonies were selected and polymerase chain reactions (PCR) were performed using forward degenerate primer CPD3FT (5'-TG(CT) GG5 (AT)(GC)5 TG(CT) TGG GC-3') and reverse primer SD3prime (5'-CAA CGG CAT TAA ACC GAA TT-3') (Gene Technologies Laboratory, Institute of Developmental and Molecular Biology, Texas A&M University, College Station, Texas), with locations indicated in Fig. 1. PCR was performed in 20 l volume containing 50-100 ng of the template DNA, 1 pmol of each primer, 5 mM KCl, 1 mM Tris HCl, pH 8.3, 1.5 mM $MgCl_2$ and 0.4 U TAQ polymerase. Negative controls included all but the template DNA. Amplification was carried out in a

thermocycler (Express or Sprint Thermal Cycler; Hybaid, Middlesex, United Kingdom) using a hot start method (94 ºC for 10 min). The amplification process consisted of initial denaturation at 94 ºC for 10 min; 30 cycles of 94 ºC denaturation for 1 min, 55 ºC annealing for 1 min, 72 ºC extension for 1 min; final 72 ºC extension for 10 min, followed by a hold at 4 ºC. All PCR products were electrophoresed through 1% agarose gels, stained with ethidium bromide and visualized by UV transillumination to confirm the expected 700 bp size of the PCR product.

The pDNA without the Bmcp1 gene insert (empty pDNA), was prepared by transformation of *E. coli* DB3.1 competent cells by the pYD52 destination vector, which confers ampicillin resistance to the *E. coli*. Unlike other strains of *E. coli*, DB3.1 cells possess a gyrase mutation, which allows growth despite the presence of the *ccd*B gene. The transformation was then plated on LB plates with 100 μg/ml of ampicillin and incubated for 15 h at 37 ºC.

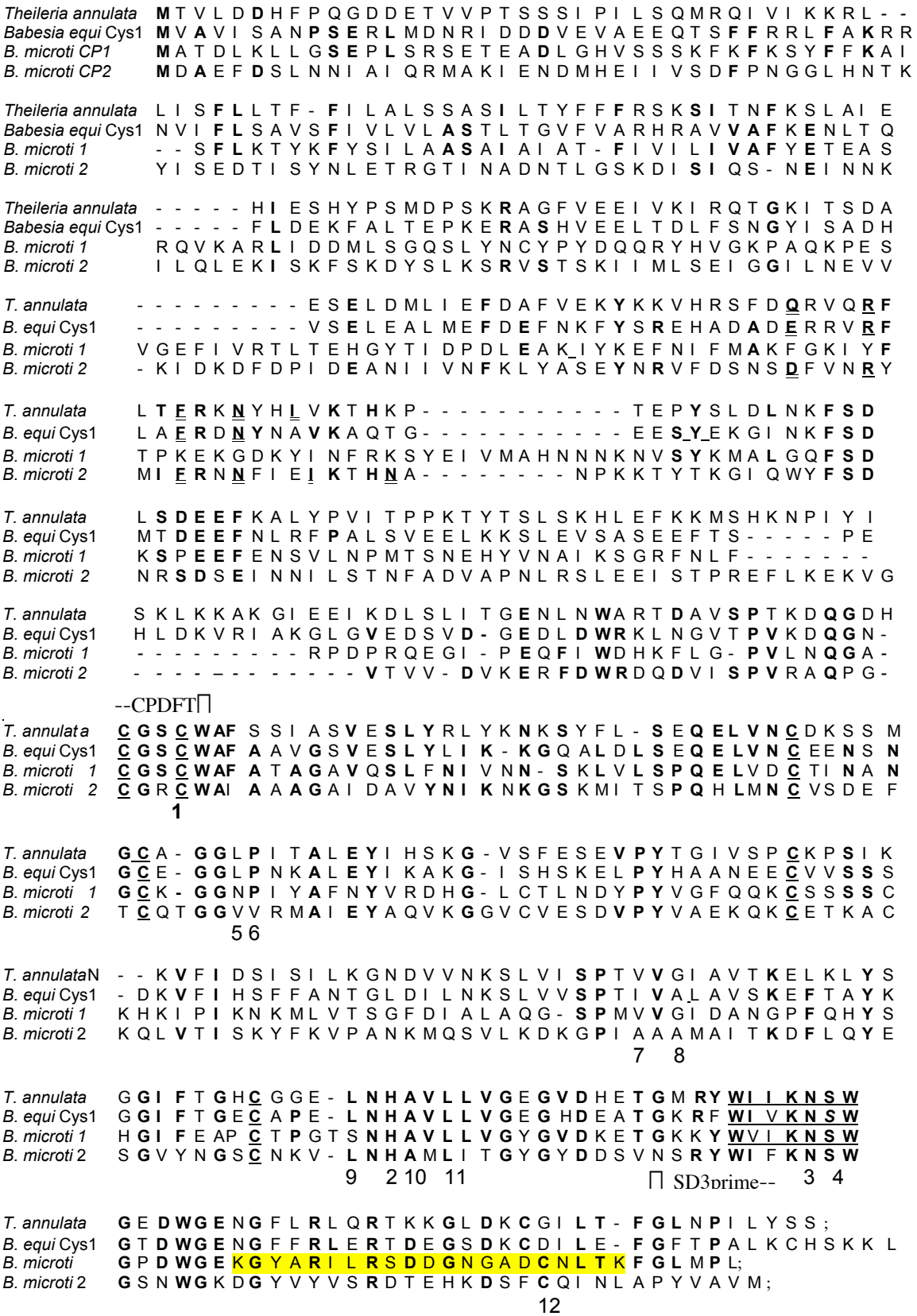
The pYD52 with Bmcp1 in DH5α *E. coli* and the empty pYD52 in DB3.1 *E. coli* were propagated by inoculating a single PCR positive colony into 6 ml of LB medium with 100 μg/ml ampicillin at 37 ºC with shaking (250 rpm) for 8 h, followed by sub-inoculation of 0.3 ml of the most turbid cultures into 149.7 ml of LB medium with 100 μg/ml ampicillin at 37 ºC with shaking (220 rpm) for 16 h. Some of the culture that was sub-inoculated was also cryopreserved as stock in 50% glycerol (flash frozen in liquid nitrogen for 30 sec. and then stored at –80 ºC).

After the cultures incubated for 16 h, Qiagen HiSpeed Plasmid Maxi Kits (Qiagen) were used to purify pYD52 with Bmcp1 from DH5α cells and empty pYD52 from

DB3.1 cells, according to protocol for future use in animals (Qiagen 12662). All batches of purified pYD52 with Bmcp1 were pooled, as were the batches of empty pYD52, and the DNA concentrations of the respective pools were determined by spectrophotometry (Biotech Photometer UV 1101, WPA). A sample of purified pYD52 with Bmcp1 was sequenced with primers located within the vector, forward primer BmPDF (5'-TAG CAG CTG TAA TAC GAC TC-3') and reverse primer BmPDR (5'-AAC TCA ATG GTG ATG GTG-3') (SIGMA- GENOSYS, The Woodlands, Texas) to confirm presence, direction, and proper frame of the insert (DNA Technologies Core Lab, Department of Veterinary Pathobiology, Texas A&M University, College Station, Texas).

The purified pDNA was ethanol precipitated and resuspended in the minimum amount of ultrapure water required for resuspension. The final concentration was determined by spectrophotometry and the pDNA was stored at –20 ºC until it was used.

Fig.1. Amino Acid Sequence of *Babesia microti* Cysteine Protease-1 Gene (CP-1) and Cysteine Protease Peptide (CP). Deduced amino acid sequences from *Babesia microti* cysteine protease genes aligned by Holman et al. (2002) with those of *B. equi* and *Theileria annulata* (GenBank Accession Nos. AF036251 and M86659). Residues of the S_1 subsite (catalytic triad members) are indicated by the numbers 1-4, critical cysteine residues are bold and underlined, and S_2 subsite residues (substrate specificity) are indicated by the numbers 5-12. Gaps were introduced to facilitate alignment (-). Residues conserved between *B. microti* and either *T. annulata* or *B. equi* are in bold type. Residues of the ERFNIN-like sequence in the proregion are in bold and double underlined. The CP sequence is highlighted. Locations of the PCR primers used to determine presence of the cysteine protease in pYD52 are indicated.


Theileria annulata
Babesia equi Cys1
B. microti CP1
B. microti CP2
B. microti 1
B. microti 2
T. annulata
B. equi Cys1
--CPDFT⌐
1
5 6
T. annulataN
7 8
9 2 10 11
⌐ SD3prime-- 3 4
12

2.2. Bmcp1 peptide (CP)

A peptide derived from Bmcp1 (CP) (Fig. 1) was synthesized by fluorenylmethoxycarbonyl (Fmoc) solid-phase chemistry employing a Millipore 9050 Plus (Perceptive Biosystems) automated synthesizer and purified by the Peptide Synthesis Core Facility, Department of Veterinary Pathobiology (Dr. Judith Ball).

2.3. Vaccine preparation

The pYD52 with Bmcp1 and empty pYD52 were adjusted to a final concentration of 2 □g/□l with Dulbecco's phosphate buffered saline (PBS, pH 7.4) (Sigma) just prior to inoculation. The peptide boost was prepared by resuspending the synthetic peptide CP in PBS (3:4 ratio by volume) and then adding Sigma Alhydrogel (13 mg/ml) (1:4 ratio by volume) to a final concentration of 2 □g/□l. The suspension was incubated at 4 ºC while turning on a DYNAL□ Rotamix for 12 h. The control for the CP peptide was prepared in the same manner as described above, excluding CP. The experimental controls received PBS only.

2.4. Injections and vaccine trial procedure

Female BALB/c mice (Harland Labs, Houston), 3-5 months of age, were used for the vaccine trials. Two vaccine trials were carried out, each consisting of five groups of four BALB/c mice. The five groups represented: 1) pYD52 with Bmcp1 (pYD52/Bmcp) followed by two pYD52/Bmcp boosts, 2) pYD52/Bmcp followed by two CP/Alhydrogel

boosts, 3) pYD52 without an insert (pYD52) followed by two pYD52 boosts, 4) pYD52 followed by two boosts of Alhydrogel only, and 5) PBS followed by two boosts of PBS.

Primary inoculations were administered intramuscularly under anesthesia. The mice were anesthetized individually prior to receiving the intramuscular injection (i.m.) of either 50 µl of pYD52 with Bmcp1 (100 µg), 50 µl of empty pYD52 (100 µg), or 50 µl of PBS into the thigh muscle. The anesthetic agent, isoflurane, was administered by inhalation using an induction chamber and anesthesia was maintained during the injection by inhalation through a facemask. All injections were administered using 30 gauge, 0.5cc syringes (ReliOn®). Boosts were administered subcutaneously (s.c.) and consisted of either 50 µl of pYD52 with Bmcp1 (100 µg), 50 µl of empty pYD52 (100 µg), 50 µl of CP (100 µg), 50 µl of the control for the peptide, or 50 µl of PBS. The first boost was administered two weeks after the primary inoculations, followed by the second boost two weeks later. Three weeks after the second boost, all mice were challenged with *B. microti* parasites by intraperitoneal inoculation. Three days prior to challenging the mice, the pre-challenge packed cell volume (PCV) was determined and plasma was collected. The PCV of each mouse was determined by collecting blood from the tail tip in heparinized microhematocrit capillary tubes (Fisherbrand®, Pittsburgh, PA), followed by centrifugation for 30 sec. (IEC MB micro hematocrit centrifuge). The mice in the first vaccine trial received a direct inoculation of $2x10^7$ *B. microti* infected erythrocytes, which were previously maintained by sub-passage in BALB/c mice until used for the live challenge. The mice in the second vaccine trial received 10^5 infected erythrocytes.

2.5. Analysis of infection

The infections were monitored daily by blood smears of blood drawn from the tip of the tail. The smears were methanol-fixed and then Giemsa-stained for microscopic examination at 1000X under oil immersion. Parasitemias were monitored by counting the number of *B. microti*-infected erythrocytes among 1000 erythrocytes. PCV was also determined at key points during parasite challenge such as when parasitemia began to rise, when parasitemia began to drop, and when mice recovered from parasitemia (corresponding to days 7, 11, and 23 for the first vaccine trial, and days 12, 15, and 28 for the second vaccine trial). After the parasitemia cleared, as determined by three consecutive days without parasite observation in blood smears, the mice were humanely euthanized. Euthanasia was carried out by placement in a CO_2 chamber, directly followed by cardiac puncture to exsanguinate the mice. Blood was collected into EDTA. Mouse plasma was collected after centrifugation of the blood at 1200 rpm for 20 minutes (Jouan GR412). The plasma was then stored frozen at –80º C until analyzed. The plasma collected from the second vaccine trial was analyzed by enzyme-linked immunoabsorbant assay (ELISA) in order to detect specific antibody activity to Bmcp1.

2.6. ELISA

Microtiter plates were precoated (75 □l/well) with a solution of Poly-L-Lysine (Sigma P2636, St. Louis, MO) in bicarbonate buffer (final pH and concentration, 9.6 and 1.6 □l/ml, respectively) and incubated for 1.5 h at room temperature (RT). The plates were washed one time with PBS/Tween-20 (0.05% Tween-20, Sigma P1379). The plates

were then coated (75 µl/well) with 1% glutaraldehyde (Sigma G5882, grade 1) and then incubated at RT for 13 min. The plates were then washed one time with wash solution (Tween-20 in 1M NaCl, 0.05% Tween-20 final concentration).

The antigenic substrate, CP peptide, was diluted to 10 µg/ml in PBS and 75 µl were added to each well except negative control wells with PBS only and positive control wells with mouse NSP4 peptide. The plates were sealed and incubated overnight at RT. The next day, the plates were rinsed twice with wash solution.

Non-specific binding was blocked by first adding 200 ul of 1M glycine in PBS to each well, and incubating for 1 h 45 min at 37 ºC. Then, the solution was flicked out of the plates and 200 µl of a 1:1 mixture of 5% blotto (non-fat dry milk in PBS) and 1% gelatin was added to each well and the plate incubated for 1 h at 37 ºC. The solution was shaken from the plates.

Starting dilutions of 1:25 were prepared for the pre- and post-vaccination plasma of each mouse in 5% blotto. The starting dilutions of the pre- and post-vaccination plasma, positive control, and negative control were carried through six serial 2-fold dilutions, with a final volume of 75 µl/well for each dilution.

The standard positive and negative control sera used for the test were the pre- and post-sera of mice inoculated with NSP4 peptide developed and previously tested by the Peptide Synthesis Core Facility, Department of Veterinary Pathobiology (Dr. Judith Ball). An additional control was 5% blotto as primary antibody. The plates were incubated for 1 h at 37 ºC. The solution was shaken out of the plates and the plates were washed seven times with wash solution.

The second antibody, Horse Radish Peroxidase (HRP)-conjugated anti-mouse antibody, was diluted 1:5000 in 5% blotto and 75 µl were added to all wells and then the plates were incubated for 1 h at 37 ºC. The solution was shaken out and the plates were washed seven times with wash solution. Tetramethylbenzadine substrate (Pierce) was added to every well (100 µl/well), incubated 8-10 min at RT, and then the reaction was stopped by adding 100 µl of 2M sulfuric acid (H_2SO_4) to every well. The absorbance was read at 450 nm.

2.7 Statistical analyses

Student's t-test was used to compare the mean parasitemias, PCVs, antibody titers, time to peak parasitemia, and time from peak parasitemia to clearance of the parasite from blood smears. P-values ≤ 0.05 are considered statistically significant.

3. RESULTS

3.1 Vaccine Trial I

3.1.1. Parasitemia

The mice in the first vaccine trial were challenged with 2 x 10^7 *B. microti*-infected erythrocytes. Infected erthrocytes were observed in Geimsa-stained blood smears of all mice beginning on day 2 post-challenge, after which 10 to 20 days lapsed before parasites were no longer detected microscopically (Fig. 2). One mouse in the control group that received pYD52 with two boosts of Alhydrogel showed a significantly lower parasitemia ($P< 0.05$) than any other mouse in Vaccine Trial I. This mouse was therefore considered an outlier and was not included in statistical analysis. No statistically significant differences were found between the peak parasitemias of any of the experimental groups when compared to the appropriate controls ($P> 0.05$, all group comparisons). The highest and lowest mean peak parasitemias were observed in experimental groups. The highest, 40.5%, was observed in the experimental group that received pYD52/Bmcp with two CP/Alhydrogel boosts. The lowest mean peak parasitemia, 30.7%, was observed in the experimental group that received pYD52/Bmcp with two pYD52/Bmcp boosts. This latter group was also determined to be clear of *B. microti* (recovered) two to three days before all other groups, as determined by three consecutive days of blood smears with no microscopically detectable parasitemia. All mice recovered within 19 days of being challenged.

3.1.2. PCV

The mean PCV of all vaccine trial groups decreased from day 0 to day 7 at a rate of approximately 1% per day (Fig. 3). From day 7 to day 11, the mean PCV dropped dramatically, approximately 2.5% per day for all groups (Fig. 3). The dramatic drop in PCV between days 7-11 correlates with the mean peak parasitemias for all groups, which occurred on day 9 (Fig. 3). The PCVs of the experimental groups, pYD52/Bmcp with two CP/Alhydrogel boosts and pYD52/Bmcp with two pYD52/Bmcp boosts, were not significantly different from those of the controls ($p>0.05$). The group that showed the least drop in PCV was the control group that received pYD52 with two pYD52 boosts. The group that showed the greatest drop in PCV was the control group that received pYD52 with two boosts of Alhydrogel/PBS. After the mice appeared to be clear of parasitemia, a final PCV was determined. The experimental group that received pYD52/Bmcp with two pYD52/Bmcp boosts showed the PCV returning to normal levels at a faster rate than all other groups (Fig. 3). The PCVs of the control group that received pYD52 with two boosts of Alhydrogel/PBS remained lower than all other groups (Fig. 3).

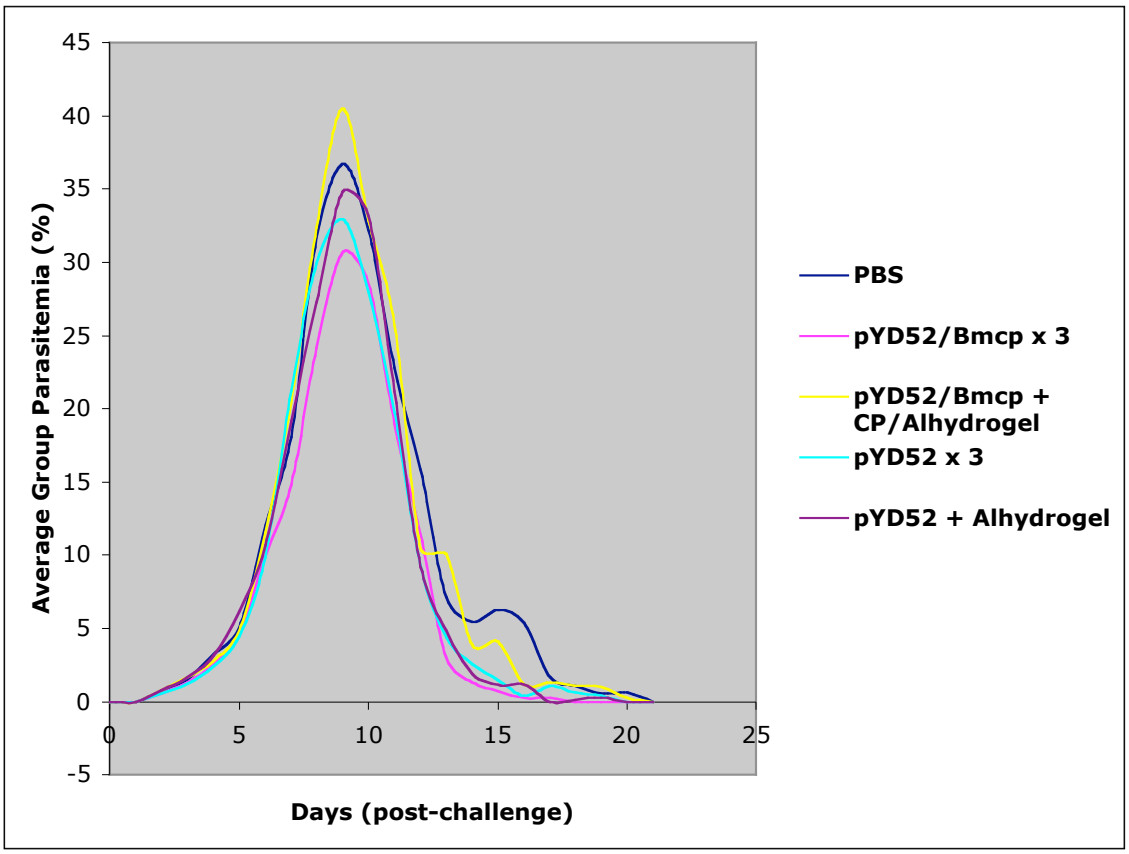


Fig.2. Vaccine Trial 1: Average Group Percent Parasitemias

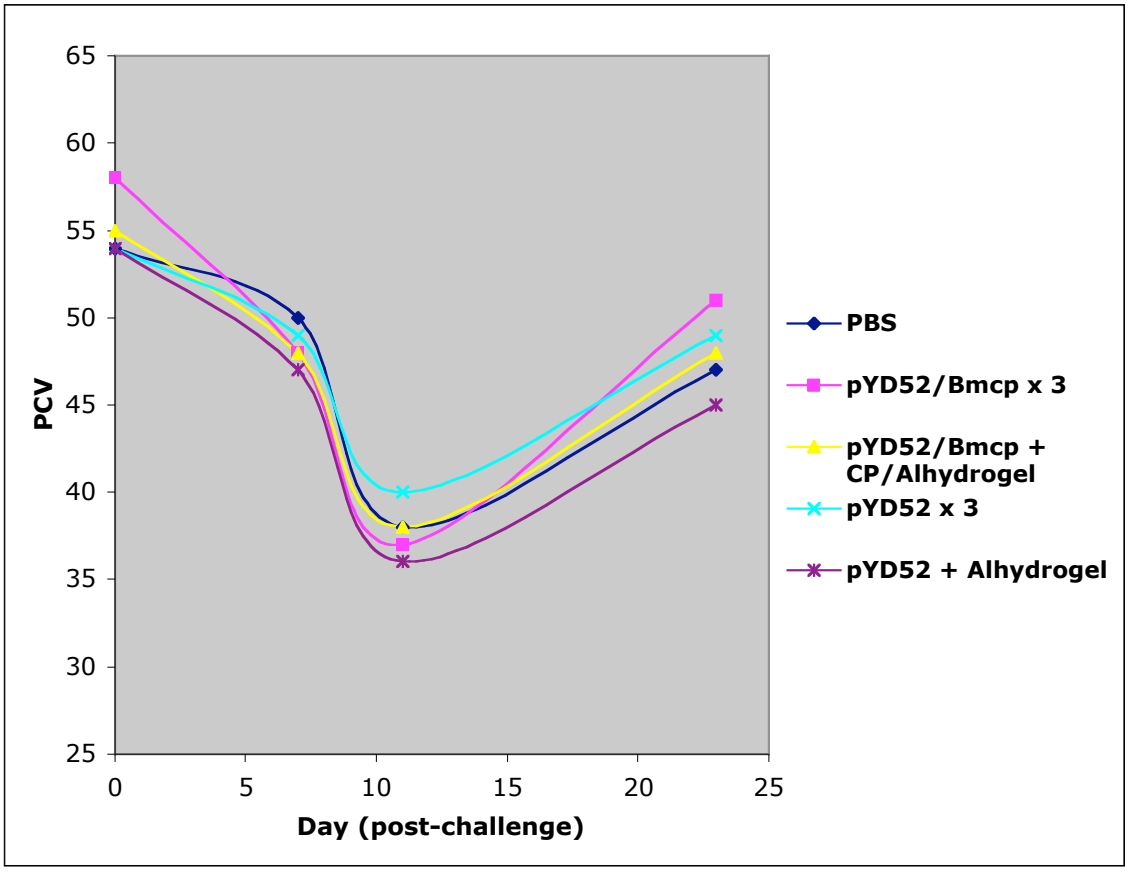


Fig.3. Vaccine Trial 1: Average Group Packed Cell Volume (PCV)

3.2. Vaccine Trial II

3.2.1. Parasitemia

The mice in vaccine trial II were challenged with 10^5 *B. microti*-infected erthrocytes. *B. microti*-infected erthrocytes were observed in Geimsa-stained blood smears of all mice beginning 4 to 7 days post-challenge, after which 15 to 21 days lapsed before parasites were no longer detected in blood smears from the mice (Fig. 4). Parasites were detected in two control groups, the control pYD52 with two pYD52 boosts and the PBS control group, 1 and 3 days respectively, before parasites were detected in other groups. One mouse in each of the 5 groups showed either no parasites or a significantly lower parasitemia in the blood smears, when compared with mice in the same group or in vaccine trial II as a whole ($P< 0.05$). These mice were therefore considered outliers and were not included in statistical analysis. The experimental control group that received pYD52 with two boosts of pYD52 was the only group with parasitemias significantly different from other groups. This group had a significantly lower mean peak parasitemia than the experimental group that received pYD52/Bmcp with two pYD52/Bmcp boosts ($P< 0.05$) and the control PBS group ($P< 0.05$). The highest mean group peak parasitemia, 18.2 %, was observed in the experimental group that received pYD52/Bmcp with two pYD52/Bmcp boosts. The lowest mean group peak parasitemia, 13.6 %, was observed in the control group that received pYD52 with two boosts of pYD52. All mice recovered within 25 days of being challenged.

3.2.2. PCV

From day 0 to day 12, the mean PCV of all the groups, except the group that received pYD52/Bmcp with two pYD52/Bmcp boosts, decreased at an average rate of 1.4 to 1.7 % per day (Fig. 5). The PCV of the group that received pYD52/Bmcp with two pYD52/Bmcp boosts decreased at an average rate of 0.8% per day. From day 12 to day 16 post-challenge, the mean PCV dropped for all groups except the PBS group. The PCV of the PBS group increased at a rate of 1% per day from day 12 to day 16. The PCV of the group that received pYD52/Bmcp with two pYD52/Bmcp boosts increased at a rate of 1.5% per day from day 12 to 16, while the PCV of the remaining groups increased at rates ranging from 0.25% to 0.75%. The low PCV values determined on day 12 correspond to the time when the mice were experiencing rising parasitemias near peak parasitemias. The rising PCV values determined on day 16 correspond to the time when the parasitemias of the mice were declining. The dramatic drop in PCV between days 7-11 correlates with the mean peak parasitemias for all groups, which occurred on day 9, post-challenge. The group that received pYD52 with two pYD52 boosts showed the least drop in PCV. The group that received pYD52 with two boosts of Alhydrogel/PBS showed the greatest drop in PCV. After no parasites were detectable microscopically in the mice, a final PCV was determined. The experimental group that received pYD52/Bmcp with two pYD52/Bmcp boosts recovered at a faster rate than all other groups (1.75% per day). The final PCV of all groups ranged between 50 to 52 %, compared to 57% at the outset of vaccine trial II (Fig.5).

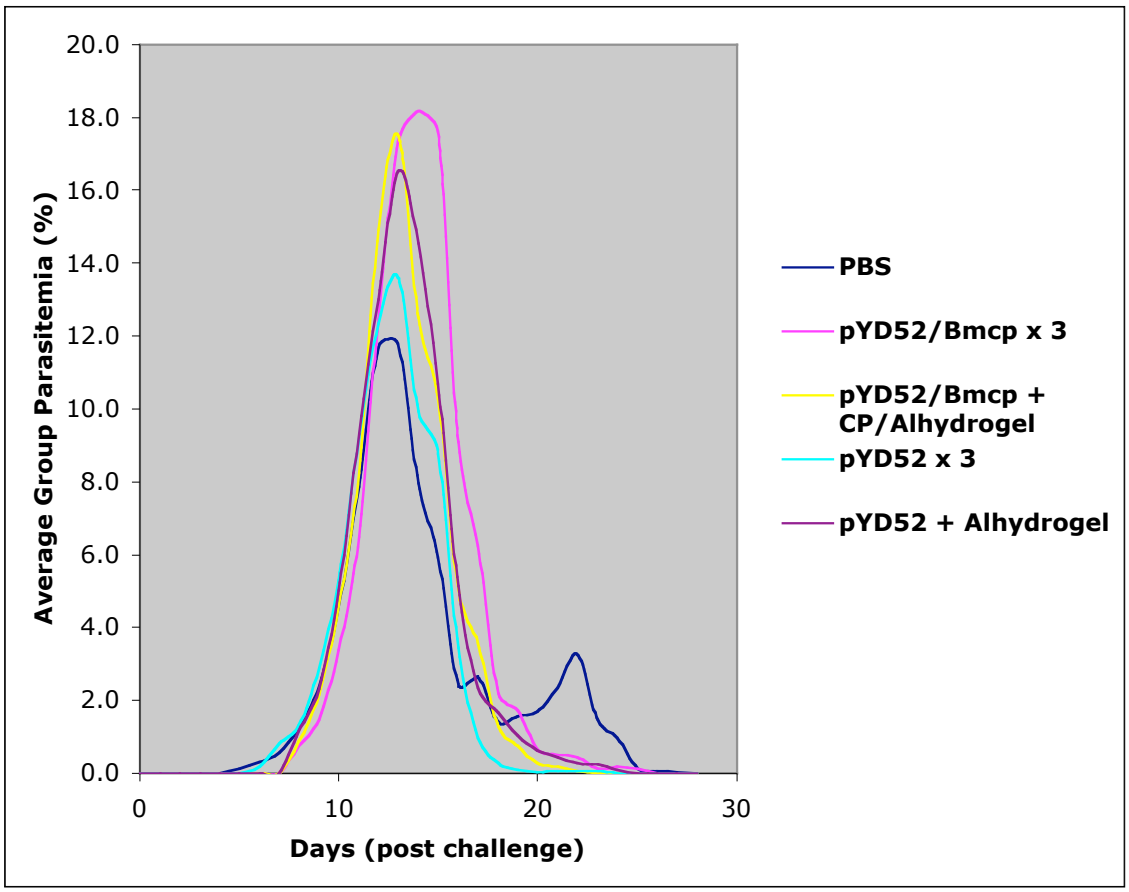


Fig.4. Vaccine Trial 2: Average Group Percent Parasitemias.

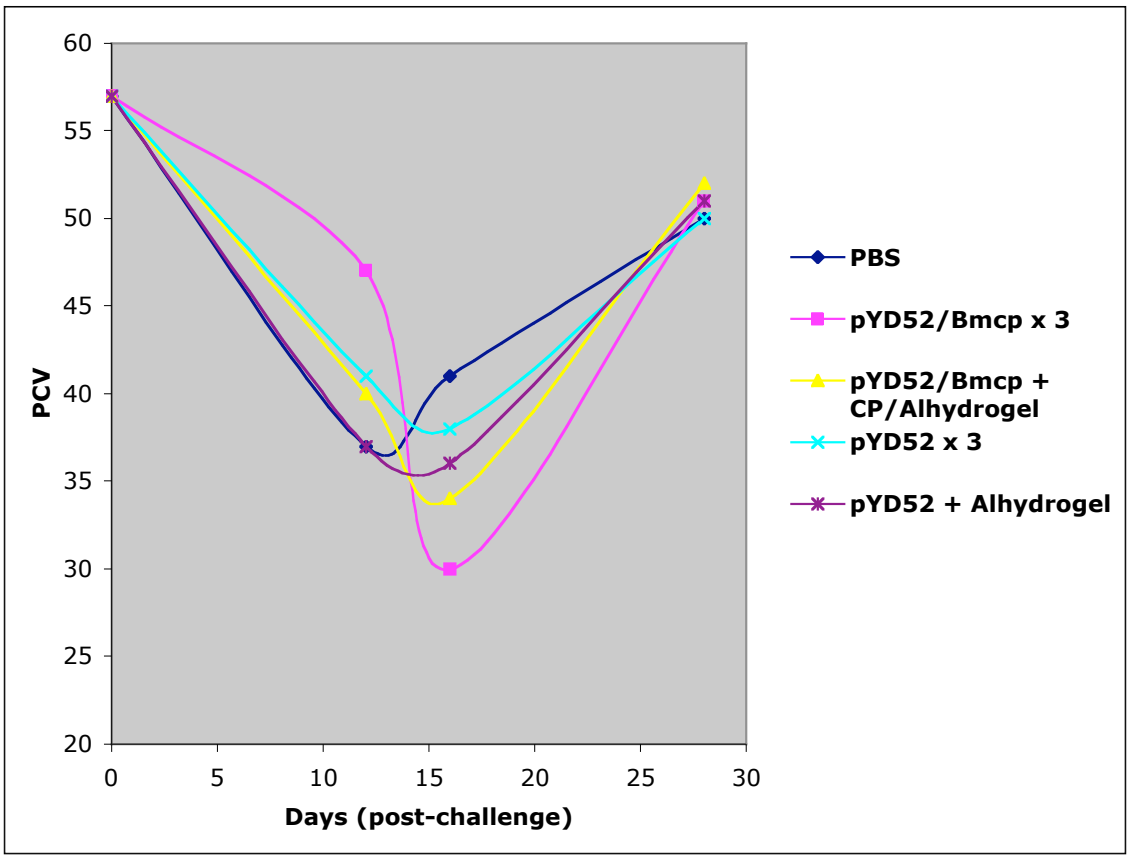


Fig.5. Vaccine Trial 2: Average Group Packed Cell Volume (PCV)

3.2.3. ELISA

The pre- and post-plasma samples from each mouse were negative for *B. microti* cysteine protease-specific antibody at all dilutions tested. The titer for the positive standard control was observed at 1:250 for each test. The negative standard control for the cysteine protease antigen and all plasma samples from the control mice were consistently negative. The standard positive and negative controls functioned properly, thus, the ELISA was carried out without error.

NOTE: The plasma samples from the first vaccine trial were not analyzed because they were deemed unfit for analysis after they were heated for an unknown amount of time in a broken freezer (The inside of the freezer was hot to the touch and condensation was observed on the lids of the samples).

4. DISCUSSION AND SUMMARY

Babesia species have a worldwide distribution, affecting a wide range of mammalian hosts (Levine, 1985). The major route of transmission is through the bite of an infected *Ixodid* tick, however, other routes of transmission could include transplacental, intrauterine, blood transfusion, organ transplant, or blood transfer during fighting (canids) (Neitz, 1956; Du Plessis and Basson, 1966; Levine, 1985; Errsenio-Jenssen et al., 1987; de Vos et al., 1994; Taboda, 1997; Phipps and Otter, 2004). *Babesia* species of major economic importance are those that cause equine and bovine babesiosis.

Equine babesiosis threatens the importation and exportation of horses and decreases the performance of horses. The United States is at risk of infection coming in from Florida, where approximately 10,000 horses are imported and exported every year. The first outbreak of equine babesiosis in the United States occurred in Florida in 1959, after infected horses were imported from Cuba (Holbrook, 1969; Knowles, 1998). The outbreak resulted in the death of 20% of the infected horses and $5 million in losses.

Bovine babesiosis caused major economic losses in the 1860's, when *B. bigemina* and *B. bovis* caused the death of over 15,000 head of cattle, in addition to weight loss and decreased milk production (Smith and Kilborne, 1893; Marquart et al., 2000). If a similar outbreak occurred in the United States today, the economic losses would amount to over a billion dollars. In order to eliminate bovine babesiosis from the United States, the Cattle Fever Tick Eradication Program (CFTEP) was put into action in the late 1800's. This program effectively cleared the United States of *B. bigemina*, *B. bovis,* and

the vector tick by mandatory dipping of all cattle in the southern United States. Today, the United States is threatened by the possible reintroduction of *Boophilus* ticks. The risk of establishment of tick populations is increased by the development of acaricide resistance among *Boophilus* populations (Corson, 2001). CFTEP "tick riders" still monitor the border between the United States and Mexico, which is endemic with *B. bovis*- and *B. bigemina*-infected *Boophilus* ticks, to apprehend stray livestock coming into the United States from Mexico. Stray and smuggled animals from Mexico can carry infected ticks. A report in 2000 noted that 50% of the cattle and 20% of the horses seized during smuggling attempts into the United States were infested with *Boophilus* ticks (Bowers, 2000). In addition, deer carrying infected ticks also pose a risk as they freely move from Mexico to the United States.

Another *Babesia* species of growing concern in the United States is *B. microti*, the primary cause of human babesiosis (Gorenflot et al., 1998). Within the last decade, the frequency of diagnosed cases has increased (Kjemtrup and Conrad, 2000). Clinical profiles of human babesiosis can range from asymptomatic to highly pathogenic and fatal. The degree of parasitosis is directly related to the efficiency of the host immune response, which can be inhibited by concurrent infection or splenectomy (Gorenflot et al., 1998).

In order to fight infection with *Babesia,* it is important for the host to mount both a cellular and humoral immune response. A humoral immune response is responsible for eliciting IgG to bind and label extracellular parasites for elimination during the early stages of infection, and later in infection, the humoral immune response stimulates

specific antibodies to the *Babesia* species (Goodger et al., 1992; Parham, 2000). The cellular immune response, specifically CD4+ T cells and the subsequent release of IFN-, are important for attacking intracellular parasites (Ikuo et al., 1994; Shimada et al., 1996). Together, cellular and humoral immune responses can help clear infection and protect against re-infection (Parham, 2000; Aguilar-Delfin et al., 2003).

Vaccines have been developed against a number of *Babesia* species with variable success (Callow, 1971; Singh et al., 1981; Wright, 1991; Bock et al., 1992; Kung'u et al., 1992; Echaide et al., 1993; Lawrence et al., 1993; Goodger et al., 1997; Ruef et al., 1997; Schetters et al., 1997; Edelhofer et al., 1998; Kumar et al., 2002b; Pipano et al., 2002). Vaccine designs have included live virulent or attenuated parasites, killed merozoites, soluble parasite antigens, and recombinant proteins. Attenuated vaccines have been developed for bovine *Babesia* species, using the method of premunization, resulting in cattle that are protected against disease but must remain carriers for life in order to maintain protection (Callow, 1971; Callow, 1979; Echaide et al., 1993; Callow et al. 1997; Edelhofer et al., 1998; Pipano et al., 2002). Therefore, the attenuated vaccine design serves to benefit regions of the world that are endemic with the infected-tick vector so infection is maintained in the herd. The downsides of the attenuated vaccine are that only endemic regions benefit, it is difficult to produce in a large scale, has a short shelf life, the blood-based vaccine may carry other pathogens, and it is strain-specific (Callow et al., 1967; Wright, 1991; Bock et al., 1995; Pipano et al., 2002; Standfast, 2003).

Killed merozoite vaccines were developed to protect against *B. equi*, but vaccine trial studies have had mixed results (Singh et al., 1981; Kumar et al., 2002b). Soluble parasite antigen- and recombinant protein- based vaccines have shown promise and are safer, have a longer shelf life, and are easier to develop than live vaccines (Goodger et al., 1987; Goodger et al., 1992; Montenegro-James et al., 1992; Schetters et al., 1997; Schetters et al., 2001). However, the protection evoked is not as dependable as with live vaccines and may require continual boosting.

The need for a vaccine that avoids a carrier state or strain specificity and induces a Th1 and Th2 immune response is evident. Such a vaccine would be beneficial to bovine and equine industries, allowing trade and importation of livestock in and out of endemic areas to occur more easily. Vaccine designs for some other parasites have targeted cysteine proteases, which may play a role in immune evasion, excystment/ encystment, cell invasion, and, in a few cases, tissue invasion (Sjid and Mckerrow, 2002). Cysteine proteases are not only known to stimulate a Th1 immune response but also elicit antibodies during natural infection (Wijffels et al., 1994; Rafati et al., 2001; Schnapp et al., 2002). When the *B. equi* cysteine protease was inhibited with E64d in culture, parasite propagation was inhibited (Holman et al., 2002). In order to demonstrate the effect of a cysteine protease vaccine on a *Babesia* species using a mouse model, a prime-boost vaccine strategy was designed (Sedegah et al., 2000; McConkey et al., 2003; Rainczuk et al., 2003; Ramiro et al., 2003; Sedegah et al., 2003; Wang et al., 2004). Of the two cysteine proteases identified in *B. microti,* the one with the most similarity to the *B. equi* cysteine protease was selected (Holman et al., 2002; Mahmoud et al., 2002). The

full coding sequence for the *B. microti* cysteine protease (CP-1) was inserted into a plasmid expression vector. It was expected that intramuscular vaccination with the pDNA encoding the cysteine protease would result in uptake and expression of the encoded protein by mouse skeletal muscle cells.

The vaccine design for this study included a primary intramuscular inoculation of pDNA encoding for *B. microti* cysteine protease, followed by two subcutaneous boosts of the same or of a cysteine protease peptide (CP) adjuvanted with Alhydrogel, with appropriate controls. Two vaccine trials were carried out with identical design except for the challenge dose of *B. microti*. The results of the first vaccine trial indicated that the challenge dose of 2×10^7 infected erythrocytes might have been too high, overwhelming the immune response of the mice. Consequently, the mice in the second vaccine trial were inoculated with 10^5 infected erythrocytes, based on the estimated number of infected erythrocytes the tick vector would naturally inoculate into the mouse (Piesman and Spielman, 1982; Homer et al., 2000). The hypothesis of the study stated that vaccination of the mice with pDNA with Bmcp1 insert and the gene products would elicit a protective immune response in BALB/c mice against *B. microti* challenge when administered using a prime-boost strategy. Analysis of the daily percent parasitemia, PCV, and seroconversion of all groups revealed that a protective immune response against *B. microti* was not elicited by this vaccine strategy. The daily percent parasitemias of the first vaccine trial showed a general trend of protection in the group that received the pDNA/Bmcp with boosts of the pDNA/Bmcp, however there was no statistical significance when compared to the controls. These results suggested that the

lack of a significant difference between the groups might have been the effect of the mice being challenged with too many parasites. However, the daily percent parasitemias after a substantially lower challenge dose in the second vaccine trial confirmed that neither vaccine protocol was effective. Furthermore, there were no significant differences among the PCV values for the experimental groups versus the control groups in either vaccine trial I or II. Finally, ELISA comparisons of the pre- and post-challenge plasma samples from the mice in the second vaccine trial revealed that neither the experimental nor the control mice produced cysteine protease-specific antibody. Cumulatively, the results of the study did not support the hypothesis.

The experimental groups that received pYD52/Bmcp with two boosts of pYD52/Bmcp may not have shown a significant immune response because the skeletal muscle cells of the mice might not have taken up the plasmid, or, uptake of the plasmid may not have elicited expression of the protein. On the other hand, the experimental group that received pYD52/Bmcp with two boosts of CP/Alhydrogel may not have stimulated a significant immune response because the CP peptide went undetected by antigen presenting cells because it was too small. The ELISA results for this latter group, specifically, were surprising because the CP peptide was adjuvanted with Alhydrogel, which is known for enhancing antigenicity, in addition to activating a Th2 response and inducing IgE and IgG (Cooper, 1994; Kenney et al., 1989). However, small peptides are often conjugated to a larger protein in order to enhance the immune recognition of the protein (Hanly et al., 1995). Moreover, a key reason why antibody titers may not have been observed in the groups that received pYD52/Bmcp is that, in retrospect, the

plasmid (pYD52) was not ideal for expression of protein in mammalian cells and is primarily used for expression in yeast (Invitrogen, 12286-019). In the future, a different expression vector should be used, specifically for expression of protein in a mammalian host. Mammalian-specific plasmids have been used to express proteins in mice, resulting in high antibody titres and protection of the host from a number of parasites, including *Plasmodium*, *Theileria*, *Leishmania*, and *Trypanosoma* species (Sedegah et al., 1994; Doolan et al, 1996; d'Oliviera et al., 1997; Alarcon et al, 1999).

Previously, Mahmoud et al. (2002) described the presence of cysteine proteases in *B. microti* that were similar to conserved cysteine proteases found in different geographic isolates of *B. equi*. Furthermore, Holman et al. (2002) showed that the cysteine protease of *B. equi* is highly antigenic and is important for propagation of the parasite. For those reasons, the cysteine protease is still a promising target for future vaccines.

The DNA prime-boost vaccine is still a promising prospect for an effective *Babesia* vaccine. A vaccine strategy utilizing a different plasmid construct for the primary inoculation, followed by boosts of recombinant cysteine protease should be investigated in future vaccine trials. Recombinant cysteine proteases have been shown to stimulate the Th1 immune response, increase rate of survival, and inhibit parasite life cycles (Wright et al., 1992; Ilg et al., 1994; Rainczuk et al., 2003; Wang et al., 2004; Fukumoto et al., 2005) and have been shown to be effective vaccines for *T. cruzi*, *L. major*, *L. donovani*, *Fasciola hepatica*, and *Ancylostoma caninum* (Wijffels et al., 1994; Engel et al., 1998; Rafati et al., 2001; Das et al., 2001; Loukas et al., 2004). The utility of

recombinant proteins as effective immunogens for babesiosis is demonstrated by a recombinant surface antigen vaccine against *B. gibsoni*, which significantly reduced the level of parasitemia in dogs (Fukumoto et al., 2005).

In summary, future research targeting cysteine protease vaccines against babesiosis should consider:

1. Choice of plasmid for expression of the protein.
2. Coupling the peptide to a carrier protein.
3. Vaccinating or boosting with recombinant cysteine protease.

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